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Microcystins in Slovene Freshwaters (Central Europe)—First Report

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ABSTRACT Cyanobacterial blooms are frequent in the North-Eastern region of Slovenia, where the agricultural activities are intensive, resulting in higher water eutrophication. In a two year monitoring program we identified eighteen blooms of cyanobacteria, fifteen being hepatotoxic by intraperitoneal mouse bioassay. The approximate LD_{100} varied from 50–1000 mg/kg (dry cell weight/animal weight) and gross pathological signs were characteristic of cyanobacterial hepatotoxins. Frequently the blooms were dominated by the most common and cosmopolitan species *Microcystis aeruginosa.* Other bloom forming species were *M. wesenbergii, Oscillatoria rubescens, Anabaena flos-aquae,* and *Aphanizomenon flos-aquae.* Using high-performance liquid chromatography (HPLC), three hepatotoxins were identified, microcystin RR being the most frequent and present in highest amounts, LR, and YR. The phytoplankton analysis revealed that several different species of cyanobacteria were present in the water bodies at the time of the blooms. Although present in various water bodies, filamentous bloom-forming cyanobacteria had no chance to proliferate in the presence of the colonial genus *Microcystis.* In individual cases we were faced with a bloom in the bloom, meaning that various *Microcystis aeruginosa* blooms were heavily contaminated with another cyanobacteria, *Phormidium mucicola* which infested the mucilage of the chroococcal species. *Nat. Toxins 5:64–73, 1997.*

Key Words: cyanobacteria; blue-green algae; hepatotoxins; toxic algal blooms

INTRODUCTION

Toxic cyanobacterial blooms have been documented in several areas of Western Europe (Skulberg et al. 1984), while in the countries of Central and Eastern Europe this phenomenon is poorly documented. In this region substantially more work has been done in the field of water bloom ecology (e.g., Bucka, 1989) and taxonomy of cyanobacteria (e.g. Komarek, 1958; 1991). Similarly to other developing countries, Slovenia is facing the problem of water eutrophication and consequently cyanobacterial blooms especially in the North-Eastern part of the country (Sedmak et al., 1994). Water eutrophication *per se* is a natural phenomenon, but is highly accelerated by human activity. The cyanobacterial blooms are the direct undesirable consequence caused by the accumulation of floating cyanobacteria (Zohary, 1985). They depend on the coincidence of three preconditions: a pre-existing cyanobacterial population, a significant proportion of the organisms having positive buoyancy, and the absence or weak water mixing that overcomes the tendency of the cells to float (Reynolds and Walsby, 1975). Since all these preconditions are fulfilled in our country, we can expect them to become a growing problem. The majority of authors point out the significance of cyanobacteria as a health hazard to humans and agricultural livestock (e.g. Carmichael and Falconer, 1993). Slovenia has few natural lakes and a great number of reservoirs and other water bodies, normally not sources of potable water, but frequently used for fishing, different recreational activities, and for livestock watering. A kill of wildstock drew our attention to the toxic cyanobacterial blooms in 1990 (Sedmak et al., 1994). As a consequence, a freshwater monitoring programme was established in 1994 in order to survey the presence of potentially toxic cyanobacteria.

The purpose of this study was to identify the toxic bloom forming cyanobacteria present in our waters and we focused on the identification of heptapeptide toxins in different species.

MATERIAL AND METHODS

The Republic of Slovenia borders with Austria and Italy, two countries belonging to the European Community, and with Hungary and Croatia, two developing countries (Fig. 1).

Field sampling

Three different samples were taken at each location where the blooms occurred.

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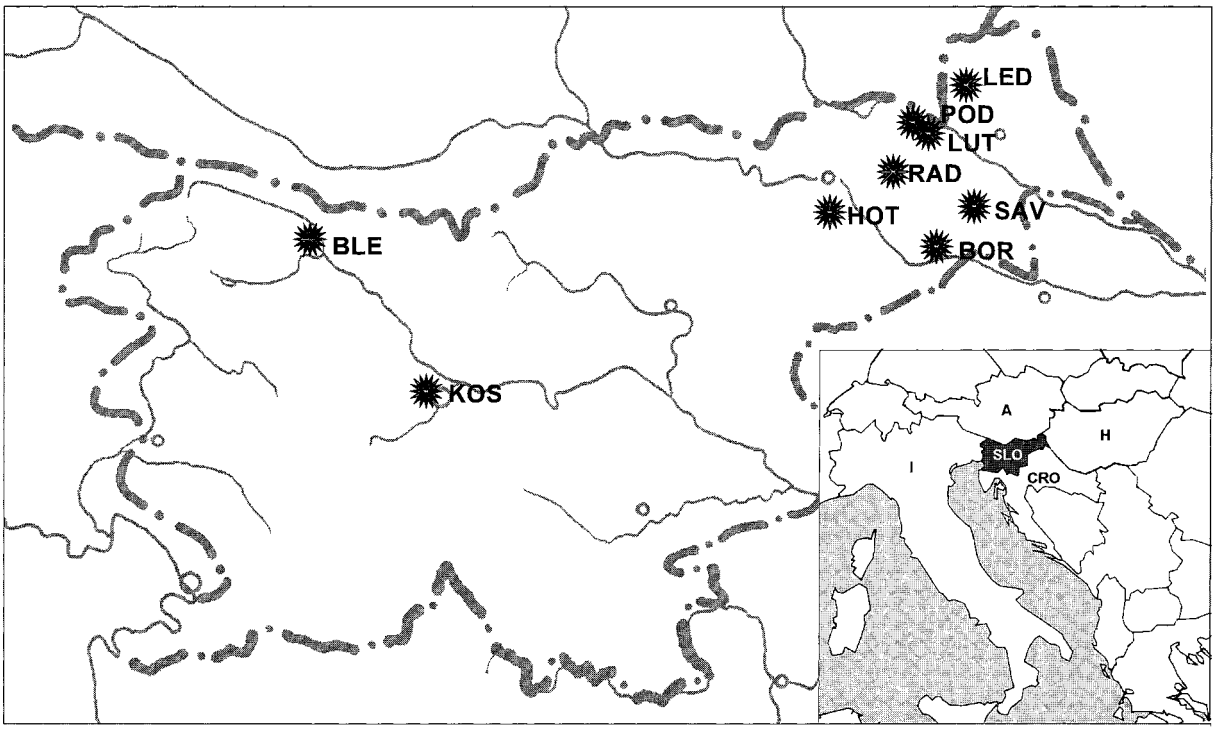


Fig. 1. Location of sampling points. BLE—Lake Bled; KOS—Koseze; LED—Ledava; POD—Podgrad; LUT—Lutverci; RAD—Radehova; HOT—Hotinja Village; SAV—Savci; BOR—Borovci.

1) Water samples. Samples for chlorophyll *a* determination were taken with a 2 L bottle 0.2–0.3 m beneath the water surface in order to avoid the blooms. 250 mL were filtered through glass microfiber Whatman GF/C filters (Whatman Ltd., Maidstone, England) and incubated in 100% methanol at 4°C for 24 hours. The pigments were extracted with hot methanol, and chlorophyll *a* was determined according to Vollenweider (1974).

2) Net samples. Qualitative 25 mesh net samples were taken as a vertical profile, preserved in 5% formaldehyde and analysed for phytoplankton species community composition. They were analysed for cyanobacterial species composition and their abundance rated using four categories: absent, present, subdominant and dominant.

3) Bloom samples. Cyanobacterial bloom samples were collected by skimming across the water surface with a 25 µm plankton net for toxin analysis. They were kept cool and brought to our laboratory. We separated larger particles and zooplankton by using different sieves. The phytoplankton samples were then concentrated by placing the material in glass cylinders under natural light. In this way, cell buoyancy was increased, the cyanobacteria floated to the surface, while the remaining algae sedimented to the bottom. The cyanobacterial scum was collected and freeze-dried.

Toxicity assay

Acute toxicity of the freeze-dried bloom material was tested by a mouse bioassay. NMRI (Naval Marine Research Institute, Lek Ljubljana) mice of both sexes weighing 20–25 g were challenged by intraperitoneal injection of 0.5–50 mg lyophilised cyanobacteria resuspended in 0.5 ml distilled water. If the mice died, necropsies were performed, and liver weights were determined as a percentage of total body weights. The livers were removed and weighed immediately after death. The approximate LD_{100} was estimated by challenging the animals with 30, 20, 10, 5, 2, 1 or 0.5 mg lyophilised cyanobacteria, selecting the dose at which the test animal died, while all animals given lower doses survived and all at higher doses died. Lethal doses were calculated considering the individual weights of the challenged mice. At least three mice per dose level were used.

Microcystin analysis

The purification of the toxins was done according to the method of Harada et. al. [1988]. Lyophilised cells (10–50 mg) were extracted three times with 5% aqueous acetic acid for 30 min while stirring. The extracts were centrifuged at 12,000 rpm, the supernatants combined and applied to 0.1 g of reversed-phase disposable extraction columns (LiChrolut Rp-18, Merck), which had been preconditioned by washing with 10 mL of methanol followed by 10 mL of water. The column containing the toxic extract was washed with 10 mL of water, followed by 10 mL of 9:1 water-methanol. Finally, the toxic fraction was eluted from the column with methanol, evaporated to dryness under a nitrogen stream, and the residue was dissolved in the buffer used for high-performance liquid chromatography (HPLC) analysis.

TABLE I. Cyanobacterial Blooms in 1994

Sampling site	Description	Date	Chl. a (µg/l) in water	Trophic cat. (OECD values)	Dominant species
Bled	Natural lake	23. 11.	2.5	Mesotrophic	*O. rubescens*
Bled	Natural lake	23. 11.	3.5	Mesotrophic	*An. flos-aquae*
Borovci	Gravel pit/fish pond	06. 07.	31	Hypertrophic	*M. aeruginosa* 90%
Hotinja V. GD	Fish pond	11. 10.	249	Hypertrophic	*M. aeruginosa* 95%
Hotinja V. LP	Gravel pit/fish pond	11. 10.	169	Hypertrophic	*M. aeruginosa*
Hotinja V. DP	Gravel pit/fish pond	11. 10.	665	Hypertrophic	*M. aeruginosa*
Koseze	Clay pit/fish pond	22. 08.	49	Hypertrophic	*M. aeruginosa*
Lutverci	Gravel pit/fish pond	06. 07.	56	Hypertrophic	*M. wesenbergii* 75%
Podgrad	Gravel pit/fish pond	06. 07.	51	Hypertrophic	*M. wesenbergii* 75%
Savci	Reservoir	07. 07.	85	Hypertrophic	*M. aeruginosa* 92%

TABLE II. Cyanobacterial Blooms in 1995

Sampling site	Description	Date	Chl. a (µg/l) in water	Trophic cat. (OECD values)	Dominant species
Bled	Natural lake	02. 02.	2.5	Mesotrophic	*O. rubescens*
Hotinja V. LP	Gravel pit/fish pond	17. 02.	324	Hypertrophic	*M. aeruginosa*
Hotinja V. GD	Fish pond	17. 02.	68	Hypertrophic	*M. aeruginosa*
Koseze	Clay pit/fish pond	20. 40.	18	Eutrophic	*M. aeruginosa* 90%
Ledavsko jezero	Reservoir	27. 10.	31	Hypertrophic	*Aph. flos-aquae*
Podgrad	Gravel pit/fish pond	27. 10.	91	Hypertrophic	*M. wesenbergii* 70%
Radehova	Reservoir	27. 10.	23	Eutrophic	*M. aeruginosa*
Savci	Reservoir	15. 09.	113	Hypertrophic	*M. wesenbergii*

The toxic fractions were separated using HPLC. All equipment was obtained from Waters, Millipore Division and consisted of a Waters 600 Multisolvent Delivery System, a Waters 616 Pump, a Waters 996 Photodiode Array Detector and Waters Fraction Collector (Milford, Massachusetts) equipped with an analytical Hibar Pre-Packed column RT 125-4 (Merck) LiChrospher 100 RP-18 (5 µm). The data was fed through an NEC Image 466 ES computer run by a Millenium 2010 Chromatography Manager (Millipore). The amounts of the toxins were estimated by comparison of the peak area of the test sample, at 238 nm, after separation with methanol: 0.05 M phosphate buffer (58:42, pH 3.0), with those of the standard samples (MC-LR, ICN Biomedicals Inc.; MC-LR R.T. 8.8 min., MC-RR R.T. 5.7 min., and MC-YR, Calbiochem R.T. 7.1 min.).

RESULTS

Incidence of Cyanobacterial Blooms

In 1994, ten cyanobacterial waterblooms were detected. The most frequent genus was *Microcystis* present in 80%; half of all blooms were formed by *M. aeruginosa.* Others were *M. wesenbergii, O. rubescens* and *An. flos-aquae.* In the following year we noticed eight blooms predominantly formed by *M. aeruginosa* in 5 cases and three individual ones by *M. wesenbergii, O. rubescens* and *Aph. flos-aquae* (Table I and II). In both years most of the blooms were dominated by one cyanobacterial species comprising over 95% of the biomass. In five cases or 28%; the blooms were a mixture of *M. aeruginosa* and *M. wesenbergii* in different proportions. *M. aeruginosa* was in most cases infested by another cyanobacteria, *Phormidium mucicola.* This is a small organism living in the mucilage of the colonial species (Fig. 2). Cyanobacterial scums were observed in summer and autumn on the surface of the water bodies. On Lake Bled the scum became visible only at the end of November. Scums in fish ponds at Hotinja Village and in Lake Bled persisted till February of the next year. In the remaining waterbodies in 1995 the blooms appeared as usual in summer or autumn. (Tables I and II).

Cyanobacteria Species Analysis

Different cyanobacterial species were present in the inspected waterbodies. Tables V and VI summarise those found during blooms at various locations. Among them are *An. flos-aquae, Aph. flos-aquae,* and *Oscillatoria agardhii,* potentially toxic filamentous bloom-forming species (locations: Bled, Savci, Hotinja Village and Podgrad—Table V; locations: Hotinja Village G.D., Ledava, Radehova, and Savci—Table VI). The majority of the *Microcystis aeruginosa* blooms were infested with the filamentous *Phormidium mucicola,* which populates the mucilage of the chroococcal host (locations: Hotinja Village and Lutverci—Table V; locations: Hotinja Village D.P., Hotinja Village L.P. and Podgrad—Table VI). The species were identified accord-

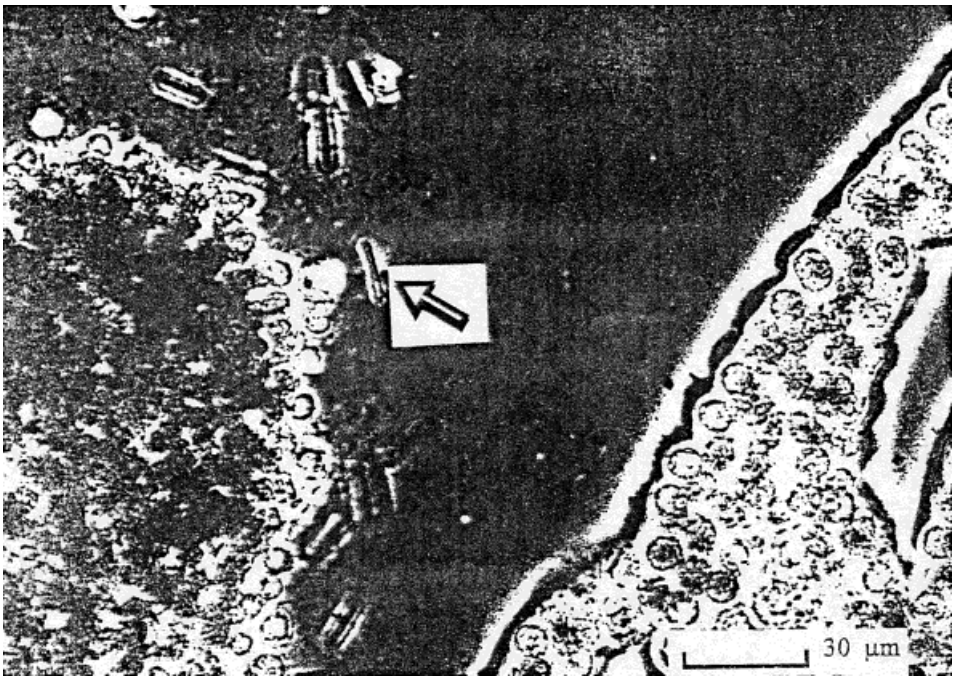


Fig. 2. Phase contrast photomicrograph of two colonies: chroococcal *M. wesenbergii* (Kom.) and *M. aeruginosa* (Kuetz.). Filaments of the cyanobacteria *Phormidium mucicola* (Hub.-Pest., Naum.) can be observed only in the mucilage of *M. aeruginosa* (scale bar 30 µm, magnification ×400).

ing to Komarek (1958; 1991), Starmach (1966) and Hindak (1981).

Bloom Toxicity

In 1994 with the mouse test 80% or eight blooms were toxic; in 1995 the figures were 87.5% or seven cases. The approximative LD_{100} ranged from 50 to 1000 mg lyophilised cyanobacteria per kg of mice. Autopsy of the dead animals revealed that their livers were dark red and markedly enlarged. Their weights increased from the 5.7% body weight of control mice to 7.1–11.4% of test mice (Table III).

Microcystins in Natural Blooms

HPLC extract analysis confirmed the presence of several microcystins in toxic blooms. We adopted the most broadly used extraction with acetic acid at pH 3. Identification of individual toxins was based on retention times and UV spectral data of standard microcystins in comparison with the elution diagrams of our samples, and on spiking the extracts with a particular standard. In this way we could identify three components that behaved like microcystin—RR, microcystin—LR, and microcystin—YR. With the comparison of the spectra extracted from different peaks with microcystin standard spectra, we located two other unidentified microcystins. Their spectra were characteristic of microcystins exhibiting the characteristic UV absorption spectrum of 3-Amino-9 methoxy-2,6,8-trimethyl-10-phenyl-4,6 decanoic acid (ADDA) with a λ_{max} at 238 nm (Fig. 3). The HPLC elution profiles of the extracts from six fish pond blooms of *M. aeruginosa* and *M. wesenbergii* were very similar. The three from Hotinja Village were almost identical to each other with 3.6 to 4.5 times more microcystin RR than LR (Fig. 4B, Table IV). The extracts from the remaining three fish ponds differed mainly in the ratio between the most frequent microcystins RR and LR (Fig. 4A and 5A, B, Table IV). The *M. aeruginosa* bloom from the water reservoir Savci, similarly to the fish pond blooms, contained both microcystins RR and LR, together with a very pronounced different peak without the spectral characteristics of microcystins (Fig. 6B). On the contrary, the *O. rubescens* bloom from the natural Lake Bled contained predominantly the microcystin YR together with another unidentified microcystin in considerable amounts (Fig. 6A). In the *M. aeruginosa* bloom from Koseze, although not toxic to mice, we identified small quantities of microcystin RR with HPL chromatography (Fig. 7).

DISCUSSION

The general findings of the present study are that toxic cyanobacterial blooms are a common phenomenon in the surface waters of Slovenia.

Slovenia is geographically a heterogeneous country. The North and the Northwest is an Alpine region with natural lakes. Due to their oligotrophic status, there are no cyanobacteria present in the water with the exception of Lake Bled, where every year cyanobacterial blooms regularly appeared (Vrhovšek *et al.* 1982). Bled is a popular tourist place, which represents a heavy burden for the water. The Western and the

TABLE III. Acute Exposure Mouse Bioassays With Bloom Samples

Sampling site	Date	Approx. LD_{100} i.p. mice (mg)[a]	Liver weight (% of body weight ± S.E.)	Isolated bloom composition
Bled	23. 11. 1994	400	10.8 ± 1.02	*O. rubescens*
Bled	23. 11. 1994	≥1500	5.7 ± 0.09[b]	*An. flos-aquae*
Borovci	06. 07. 1994	90	7.2 ± 0.33	*M. aeruginosa* 90% *M. wesenbergii* 10%
Hotinja V. DP	11. 10. 1994	100	7.1 ± 0.21	*M. aeruginosa*
Hotinja V. GD	11. 10. 1994	250	8.8 ± 0.51	*M. aeruginosa* 95%
Hotinja V. LP	11. 10. 1994	50	9.1 ± 0.37	*M. aeruginosa*
Koseze	22. 08. 1994	≥1500	5.3 ± 0.11[b]	*M. aeruginosa*
Lutverci	06. 07. 1994	160	7.3 ± 0.12	*M. wesenbergii* 75% *M. aeruginosa* 25%
Podgrad	06. 07. 1994	350	7.1 ± 0.08	*M. wesenbergii* 75% *M. aeruginosa* 25%
Savci	07. 07. 1994	90	7.5 ± 0.03	*M. aeruginosa* 92% *M. wesenbergii* 8%
Bled	02. 02. 1995	500	7.2 ± 0.19	*O. rubescens*
Hotinja V. G.D.	17. 02. 1995	1000	8.1 ± 0.40	*M. aeruginosa*
Hotinja V. L.P.	17. 02. 1995	500	9.4 ± 0.22	*M. aeruginosa*
Koseze	20. 04. 1995	1000	10.7 ± 0.39	*M. aeruginosa* 90%
Ledavsko jezero	27. 10. 1995	≥1500	5.4 ± 0.10[b]	*Aph. flos-aquae*
Podgrad	27. 10. 1995	1000	7.1 ± 0.13	*M. wesenbergii* 70% *M. aeruginosa* 30%
Radehova	27. 10. 1995	500	11.4 ± 0.18	*M. aeruginosa*
Savci	15. 09. 1995	500	8.8 ± 0.08	*M. wesenbergii*

[a]mg dry weight of lyophilised cells/kg body weight of mice, N ≥ 3.
[b]In the range of control mice.

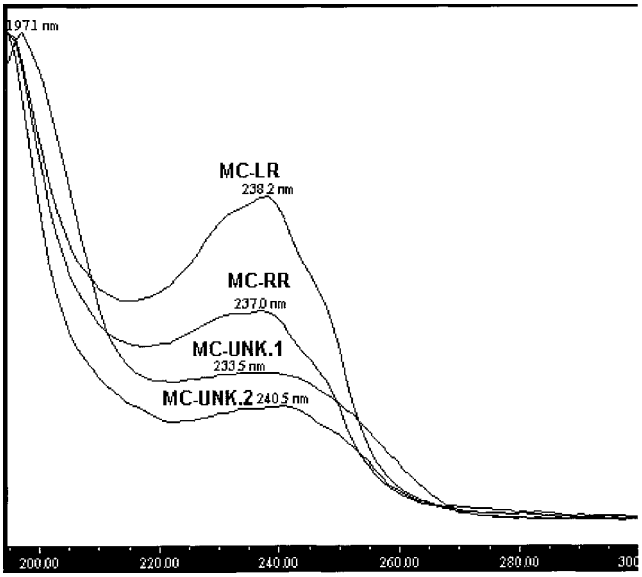


Fig. 3. UV spectra of four most frequent peaks; two spectra are identified as microcystin-LR and microcystin-RR while MC-Unk.1 and MC-Unk.2 are proposed as unidentified microcystins.

Southern parts of the country are in the Karst region with water reservoirs, where the cyanobacterial blooms were not noted. The North-Eastern region has a continental climate with hypertrophic water bodies because of intensive agricultural activities and therefore the most frequent blooms (Fig. 1).

Bled is a relatively deep lake of 30 m with characteristic thermal stratification. The cyanobacterial blooms, usually *O. rubescens* or *An. flos-aque* (Vrhovšek *et al.* 1982; Vrhovšek *et al.* 1985) and occasionally *Aph. flos-aquae* (Sedmak and Kosi, 1991), have been observed. The bloom of *O. rubescens* is usually retained in the metalimnion layer most of the time, and appears at the surface only when stratification is destroyed in the colder months of the year. Such was the case in November 1994, when two blooms were observed. At the time of sampling, the red bloom of *O. rubescens* was present in the western part and the blue green bloom of *An. flos-aquae* in the eastern part of the lake, with regions of bloom mixing. The bloom of *O. rubescens* persisted till the end of February of the next year and was toxic, not an unusual phenomenon (Jacoby et al. 1994), while the *An. flos-aquae* disappeared by the end of the year and was not toxic. Because of the stratification due to the depth and restoration activities, Lake Bled differs markedly from all other inspected water bodies. The HPLC elution profile was unique and for the only time the main peak had the retention time and spectral characteristic for microcystin YR. We identified only another peak, possibly a microcystin as judged from UV spectra. Toxic *O. rubescens* blooms have already been detected in Italian freshwater reservoirs, where

TABLE IV. Microcystin Contents in Cyanobacterial Blooms From Different Locations

	Microcystin contents (mg/g lyophilized bloom[a])				
Sampling site	MC-RR	MC-YR	MC-UNK.1[a] eqv. MC-LR	MC-LR	MC-UNK.2[a] eqv. MC-LR
1994					
Bled	0	2.88	0	0	0.55
Borovci	0.23	0	0	0.52	0.01
Hotinja V. DP	2.72	0	0	0.75	0
Hotinja V. GD	0.94	0	0	0.21	0
Hotinja V. LP	2.75	0	0	0.68	0
Koseze	0.16	0	0	0	0
Lutverci	0.31	0	0	0.16	0
Podgrad	0.48	0	0	0.06	0
Savci	18.50	0	0	0.85	0
1995					
Hotinja V. GD	1.40	0	0.28	0.32	0
Hotinja V. LP	2.00	0	0	0.48	0

[a]Estimated as microcystin LR equivalents from MC-LR calibration curve.

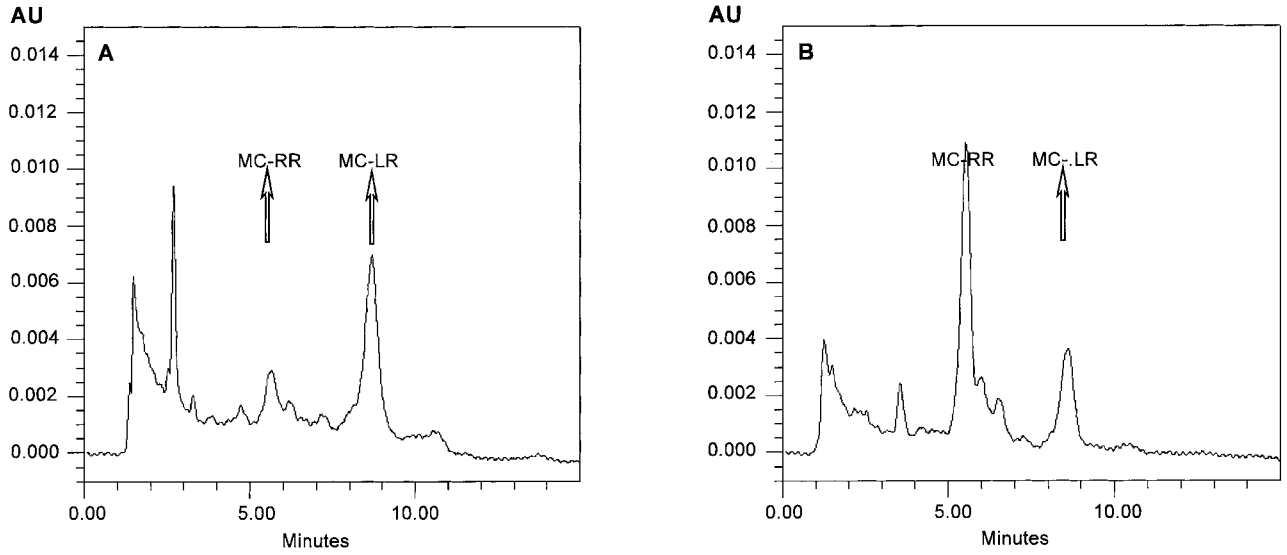
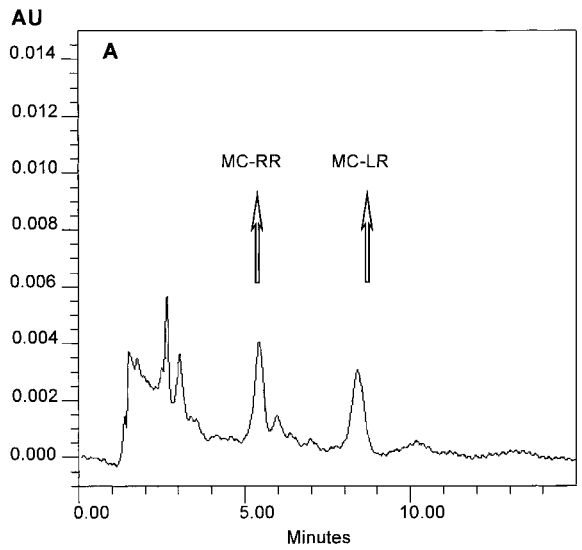


Fig. 4. High performance liquid chromatogram of the methanol eluate of the cell fraction after clean-up with RP-Silica gel cartridge. **A:** *M. aeruginosa* bloom extract from fish pond Borovci 06.07.1994. **B:** *M. aeruginosa* bloom with *Phormidium mucicola* in the mucilage from fish pond Hotinja Village L.P. 11.10.1994.

unidentified toxins produced hepatic lesions in experimental animals (Loizzo et al. 1988). In 1992 basically the same group of authors published an article claiming the presence of microcystin-like toxins in different blooms of the genus *Oscillatoria.* The main toxins were RR-like from a mixed bloom of *Oscillatoria tenuis* plus *O. rubescens,* YR-like from an *O. rubescens* and from another mixed *Oscillatoria* bloom (Bruno et al. 1992). It seems that microcystin YR could be a characteristic toxin for *O. rubescens.*

The reservoir Savci exhibits various differences from Lake Bled. It is a shallow, up to 4 m deep water reservoir surrounded by tilled fields. The cyanobacterial scum was dominated by *M. aeruginosa,* although *M. wesenbergii* was present in considerable amounts. This bloom contained the highest content of the toxins microcystin-RR at 18.5 mg/g and microcystin-LR at 0.85 mg/g dry weight of cyanobacterial cells (Fig. 6B and Table IV). Although the scums were regularly present, the toxicity determined by the mouse test and the HPLC detected toxin contents were the highest, there are very seldom fish kill problems in this reservoir. This could be attributed to the regular draining of the upper layer containing the cyanobacterial bloom from this surface-discharge lake and, as a consequence, bloom lysis could not take place in the reservoir. On the other hand, a sudden



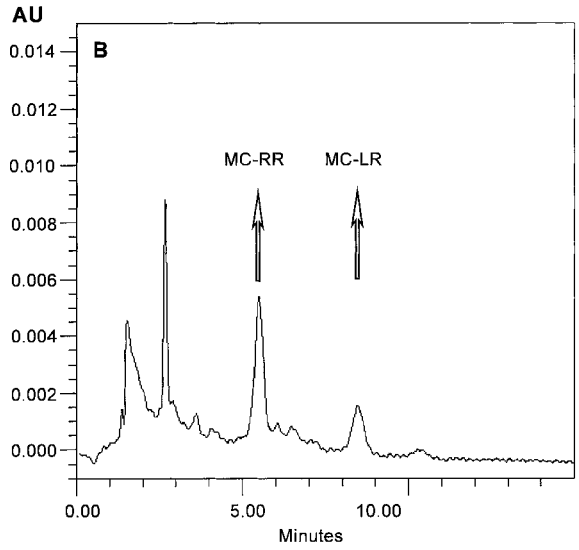
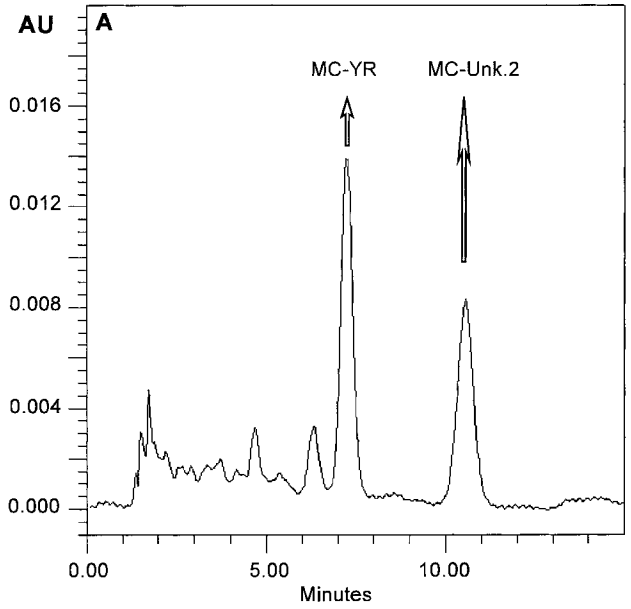


Fig. 5. High performance liquid chromatogram of the methanol eluate of the cell fraction after clean-up with RP-Silica gel cartridge. **A:** Mixed bloom of *M. wesenbergii* (75%) and *M. aeruginosa* (25%) from fish pond Lutverci 23.11.1994. Only in the mucilage of *M. aeruginosa* could we find filamentous *Phormidium mucicola.* **B:** Mixed bloom of *M. wesenbergii* (75%) and *M. aeruginosa* (25%) from fish pond Podgrad 06.07.1994. No filamentous cyanobacteria were found in the mucilage.



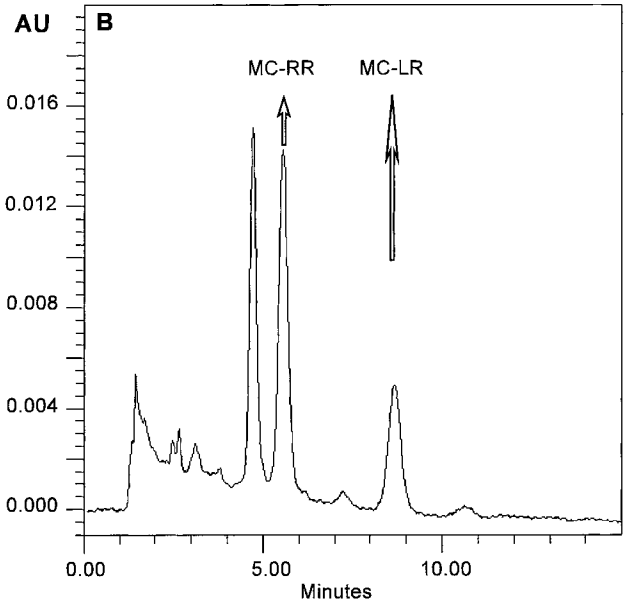


Fig. 6. High performance liquid chromatogram of the methanol eluate of the cell fraction after clean-up with RP-Silica gel cartridge. **A:** Bloom of *O. rubescens* from the natural Lake Bled 23.11.1994. **B:** Mixed bloom of predominantly *M. aeruginosa* (92%) from the water reservoir Savci 07.07.1994. No filamentous cyanobacterial species were found in the mucilage.

bloom lysis occurred in Hotinja Village D.P. in 1994, along with a massive fish kill.

The examined gravel pit fish ponds exhibited very similar elution profiles and toxin composition. All three water bodies in Hotinja Village exhibited practically identical elution diagrams with both microcystins RR and LR present (Fig. 4B and Table IV). At all three locations the dominant species was *M. aeruginosa* with *Phormidium mucicola* in the mucilage. We cannot exclude the filamentous *Phormidium mucicola* as a toxin producer, but due to the small, few-celled 1.5 µm thick filaments they do not represent a substantial biomass in comparison to the large chroococcal colonies of the host. Two of the fish ponds, Hotinja Village D.P. and L.P., are situated close to each other, surrounded by fields, and therefore heavily exposed to agricultural run-off. The third, Hotinja Village G.D., is in the centre of the village and is fed by domestic effluents and runoffs from a nearby farm. Similarly, two fish ponds in Lutverci and Podgrad 2.2

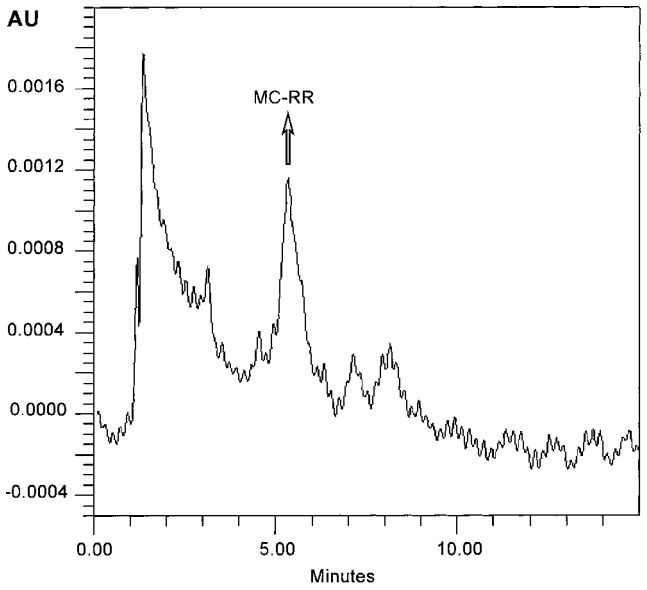


Fig. 7. High performance liquid chromatogram of the methanol eluate of the cell fraction after clean-up with RP-Silica gel cartridge. Bloom of *M. aeruginosa* found nontoxic by mice bioassay from the fish pond Koseze 22.08.1994.

km apart from each other are also exposed to similar environmental conditions: the vicinity of a hog farm in an intensive agricultural area. The bloom species composition was identical in both locations with a similar toxin content (Fig. 5A and B; Table IV). The third major inspected fish pond location was Borovci. At this location we can also find the most frequent toxins microcystin RR and LR, with the difference that the latter prevailed (Fig. 4A, Table IV).

All three major fish pond locations form a triangle with the edges 26–32 km from each other. The water bodies are visited mainly by local fishermen so that the chances of cyanobacteria dissemination by means of sport activities are very little. The only living category that could be actively involved in dissemination are birds. Microscopical examination reveals, that the mucilage of *M. aeruginosa* may be populated by *Phormidium mucicola,* that this never occurs in *M. wesenbergii,* although in mixed blooms (location: Lutverci, 1994) both *Microcystis* species were closely packed together (Fig. 2). The main dissemination of toxic cyanobacteria cannot be by means of birds or sport activities, because there would be many more similarities in the species composition, specifically of *Phormidium* cells that may heavily populate the mucilage of *Microcystis aeruginosa* (Tables V and VI). For instance, there is a clear difference between Lutverci and Podgrad, two water bodies close together, with the same fishermen population, presumably also the same animal visitors, and both with a mixed *Microcystis* bloom in 1994. *Microcystis aeruginosa* from Lutverci was populated by *Phormidium mucicola* while the same species from Podgrad was not (Table V). This means that *M. aeruginosa* from both locations, although with similar elution profile, could not originate from the same clone. Many more similarities in cyanobacterial species composition could be found in locations in immediate proximity, such as Hotinja Village. All three fish ponds are located in 1 square kilometre and here a direct transfer of cyanobacterial material from one fish pond to another is more probable (Table V and VI). Therefore, in our opinion the prevailing causes for the similarity of cyanobacterial toxic blooms in the fish ponds are environmental factors and not physical dissemination. Another examined fish pond was Koseze in the capital of Ljubljana. At this location cyanobacterial blooms are also frequent. HPLC analysis of the lyophilised bloom material from 1994 demonstrated microcystin RR although in low concentrations (Fig. 7). However when it was tested on mice, it gave a negative result, and was thus described as nontoxic. The next year, the same species in the location exhibited a higher toxicity (Table III).

In most cases we are faced with the same species that will form blooms also in the following year. A significant change was the disappearance of *M. aeruginosa* and proliferation of *M. wesenbergii* in the Savci reservoir in 1995. After the bloom lysis in 1994 in the fish pond Hotinja Village D.P., there was few cyanobacteria present the following year. Relatively small number of otherwise very abundant *M. aeruginosa* and the disappearance of all other cyanobacterial species raises the suspicion that the bloom was lysed by inflow of unidentified algicide into the water.

Toxicity evaluation *in vivo* indicated the presence of hepatotoxins. As illustrated in Table 3, the liver weights of the mice treated with toxic cyanobacteria accounted for 7.1–11.4% of the total body weight, as compared to 5.2–5.8% in control mice. There are some differences between the toxicity *in vivo* (Table III and IV) and the calculated toxin contents from the HPLC elution diagrams. These could be attributed to the following facts. The lethal doses of the lyophilised blooms were approximately estimated in order to sacrifice as few animals as possible. Bloom samples were extracted by acetic acid at pH 3. The pH of extraction and also the elution conditions from the reverse phase column are quantitative only for some toxins, while others could be underestimated or even undetectable such as the neurotoxic compounds. The most frequent and abundant toxin in our waters, microcystin RR, under the mentioned conditions is underestimated (Tsuji et al. 1994).

CONCLUSIONS

Different cyanobacterial species encounter favorable conditions to form toxic blooms in Slovene freshwaters. Agricultural run-off seems to be the major cause of nutrient input into surface waters, favoring cyanobacterial blooms. Two species in particular, *M. aeruginosa* and *M. wesenbergii,* are to a great extent responsible for toxic blooms. Another small cyanobacterium was found in the mucilage of the toxic *M. aeruginosa* colonies. This filamentous species, *Phormidium mucicola,* abundantly and exclusively populates the muci-

TABLE V. Cyanobacteria Species Abundance and Composition During Bloom Conditions in 1994 *

Species	Location—abundance								
	Bled	Borovci	Hotinja V. D.P.	Hotinja V. G.D.	Hotinja V. L.P.	Koseze	Lutverci	Podgrad	Savci
Cyanophyta									
Anabaena flos-aquae (Lyngb.) Breb.	5	—	—	—	3	—	—	—	—
Aphanizomenon flos-aquae (L.) Ralfs	—	—	1	3	1	—	—	—	1
Chroococcus limneticus Lemm.	—	—	—	—	—	—	1	—	—
Gomphosphaeria aponina Kuetz.	—	1	—	—	—	—	—	—	—
Microcystis aeruginosa Kuetz.	—	5	5	5	5	5	5	3	5
Microcystis wesenbergii Kom.	—	1	—	—	—	—	3	5	3
Oscillatoria agardhii Gom.	—	—	—	—	—	—	—	1	—
Oscillatoria limnetica Lemm.	—	—	1	—	—	—	—	—	—
Oscillatoria rubescens DC.	5	—	—	—	—	—	—	—	—
Oscillatoria sp.	—	—	—	—	—	—	1	—	—
Oscillatoria subtilissima Kuetz.	—	1	—	—	—	—	—	—	—
Phormidium mucicola Hub.-Pest. et Naum.	—	—	3	3	3	—	3	—	—
Pseudanabaena constricta (Szaf.) Laut.	—	1	—	—	—	—	—	—	—
Synechocystis sp.	1	—	—	—	—	1	—	—	—

*Legend: — absent, 1 present, 3 subdominant, 5 dominant.

TABLE VI. Cyanobacteria Species Abundance and Composition During Bloom Conditions in 1995*

Species	Location—abundance							
	Hotinja V. D.P.	Hotinja V. G.D.	Hotinja V. L.P.	Koseze	Ledava	Podgrad	Radehova	Savci
Cyanophyta								
Anabaena flos-aquae (Lyngb.) Breb.	—	—	—	—	—	—	1	—
Aphanizomenon flos-aquae (L.) Ralfs	—	—	—	—	5	—	1	1
Chroococcus turgidus (Kuetz.) Naeg.	—	—	—	1	—	—	—	—
Gomphosphaeria aponina Kuetz.	—	—	—	—	1	—	—	—
Microcystis aeruginosa Kuetz.	3	5	3	5	—	3	3	—
Microcystis wesenbergii Kom.	—	—	—	1	—	5	—	5
Oscillatoria agardhii Gom.	—	1	—	—	—	—	—	—
Oscillatoria sp.	1	—	—	—	—	—	—	—
Oscillatoria splendida Grev.	—	—	—	1	—	—	—	—
Phormidium mucicola Hub.-Pest. et Naum.	1	3	—	—	—	3	—	—
Synechocystis sp.	—	—	—	1	—	—	—	—

*Legend: — absent, 1 present, 3 subdominant, 5 dominant.

lage of *M. aeruginosa*, even in mixed blooms with *M. wesenbergii*. We cannot exclude *Phormidium mucicola* as a toxin producer, but the biomass of this bloom in the bloom is small. Microcystin RR was the most frequent hepatotoxic peptide in inspected water bodies. In seems that microcystin YR is somehow characteristic for *O. rubescens* blooms. In our opinion, the main factors for toxic bloom similarities are environmental factors and not physical transfer of toxic clones.

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